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Posttranslational Modifications of p53: Upstream Signaling Pathways

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Summary

The p53 tumor suppressor is a tetrameric transcription factor that is posttranslational modified at >20 different sites by phosphorylation, acetylation, or sumoylation in response to various cellular stress conditions. Specific posttranslational modifications, or groups of modifications, that result from the activation of different stress-induced signaling pathways are thought to modulate p53 activity to regulate cell fate by inducing cell cycle arrest, apoptosis, or cellular senescence. Here we review recent progress in characterizing the upstream signaling pathways whose activation in response to various genotoxic and non-genotoxic stresses result in p53 posttranslational modifications.

1. Introduction

Maintenance of genome integrity is critical to the well being of multicellular organisms that have evolved elaborate mechanisms to monitor genome integrity and respond to a variety of environmental and cellular stresses that can disrupt the genome either directly by causing DNA damage or indirectly through disruption of normal cellular processes that involve DNA. Critical to the process of maintaining genome integrity in higher organisms is the p53 tumor suppressor, which serves to integrate signals from various DNA integrity and environmental stress-sensing signaling pathways (Fig. 1) (Wahl and Carr, 2001; Vousden and Lu, 2002; Oren, 2003). Human p53 is a 393 amino acid polypeptide that functions as a homotetrameric transcription factor to

control cell cycle progression, cellular senescence, the induction of apoptosis, and DNA repair. Genomic approaches have shown that human p53 induces or inhibits the expression of more than 150 genes including *CDKN1A* (*p21, WAF1, CIP1*), *GADD45*, *MDM2*, *IGFBP3*, and *BAX* (Sax and El-Deiry, 2003). The arrest of cells in G_1 near the border of S phase is accomplished primarily through transcriptional induction of the cyclin kinase inhibitor $p21^{Waf1}$, and cell cycle arrest is thought to allow time for the repair of DNA damage or recovery from other cellular insults. p53 also modulates DNA repair processes either directly or through the induction of repair genes (Smith and Seo, 2002; Cline and Hanawalt, 2003). The induction of cellular senescence in response to oncogene activation also involves p53-mediated accumulation of $p21^{Waf1}$, but the role of p53 in mediating senescence is not fully understood (Itahana et al., 2001). p53 mediated apoptosis involves the induction of a number of genes that may mediate the release of cytochrome c from mitochondria and leads to the activation of caspases (Vousden and Lu, 2002). Recently, it has been shown that p53 can itself directly interact with the mitochondrial membrane leading to cytochrome c release and the initiation of apoptosis (Mihara et al., 2003).

p53 normally is a short-lived protein that is rapidly degraded through ubiquitin mediated pathways and therefore is present at low levels in unstressed mammalian cells. In response to both genotoxic and non-genotoxic stresses, it becomes stabilized and accumulates in the nucleus where it binds to specific DNA sequences (Wahl and Carr, 2001; Vousden and Lu, 2002; Sax and El-Deiry, 2003) and also interacts directly with a number of other cellular and viral proteins (Fig. 2). Competition between repair proteins and damage sensors, as well as cell type-specific thresholds for initiating apoptosis may in part determine cellular fate. Stabilization of the p53

protein and regulation of its interaction with DNA and other proteins is regulated by posttranslational modifications, primarily phosphorylations and acetylations. p53 can be phosphorylated at at least 15 serines or threonines located primarily near the N or C terminus of the polypeptide chain, and it may become acetylated at a half-dozen lysines in its C-terminal domain. Before reviewing the major stress-induced signaling pathways that lead to these modifications, we first briefly review the structure of human p53 and its posttranslational modifications.

2. Structure of human p53

The structure of the intact, 393 amino acid p53 protein (Fig. 2) has proved difficult to study as the overall size of the tetrameric, p53-DNA complex, combined with its intrinsic flexibility, so far has prevented determination of its structure at high resolution (Kaku et al., 2001; Kaeser and Iggo, 2002). Only about 60% of the molecule is folded into compact domains, with the remainder forming flexible linkers or tails. These disordered regions contain most of the sites of posttranslational modification and are the loci for interactions with the many proteins with which p53 associates (Fig. 2). The N-terminal region (amino acids 1 - 101) is unstructured in solution, but residues 17-28 form an 'Y-helix upon binding to Mdm2 (Kussie et al., 1996). Residues 1-42 are required for transactivation activity and interact with the transcription factors

TFIID, TFIIH, several TAFs, the histone acetyltransferases CBP/p300, and possibly PCAF. Residues 11-26 are reported to function as a secondary nuclear export signal (Zhang and Xiong, 2001), while residues 63-97 comprise a proline-rich SH3 domain required for interaction with the Sin3 corepressor (Zilfou et al., 2001) and other proteins required for the induction of apoptosis. The structure of the DNA binding domain (DBD), residues 102-292, in complex with DNA, has been determined by x-ray crystallography (Cho et al., 1994) and NMR analysis (Rippin et al., 2002); the structure of the tetramerization domain (aa 325-356) also has been determined by both X-ray and NMR techniques (Clore et al., 1995; Jeffrey et al., 1995). A nuclear export signal that is masked in tetramers is located within the tetramerization domain, and the major nuclear localization signal is located with residues 312-324. The C-terminal 30 amino acids confer a structure-specific DNA binding capability to p53 (Palecek et al., 1997; Mazur et al., 1999), and mutual interference between sequence-specific and structure-specific DNA binding has led to proposed regulatory roles for the C-terminal domain (Hupp and Lane, 1994; Anderson et al., 1997). However, recent structural and functional studies (Ayed et al., 2001; Espinosa and Emerson, 2001; Klein et al., 2001) raised questions regarding the mechanism of p53 activation and the role of the C-terminal domain in regulating p53 activity (Ahn and Prives, 2001; Kim and Deppart, 2003).

3. p53 Posttranslational Modifications

Exposure of normal cells to either genotoxic agents or non-genotoxic stresses results in

the phosphorylation of p53 at approximately 15 serines or threonines in both the N and C terminus and acetylation at about a half-dozen lysines in the C terminus of the p53 polypeptide (Fig. 2) (Appella and Anderson, 2001; Anderson and Appella, 2003). At the N terminus, human p53 becomes phosphorylated at serines 6, 9, 15, 20, 33, 37, 46 and threonines 18, 55, and 81. Serines 33, 37, 46, and 392 are more efficiently phosphorylated after exposure to UV or adryamycin (ADR), an anti-cancer agent that inhibits topoisomerase II, than to ionizing radiation (IR); in contrast, phosphorylation of Thr18 is stronger in response to IR and ADR than to UV light (Saito et al., 2003). Phosphorylation of serines 15, 20, and 37, after either IR or UV light, increases the stability of p53 (Shieh et al., 1997; Chehab et al., 1999). At the C terminus, phosphorylation at Ser315 is induced by IR, UV, or ADR, while phosphorylation at Ser392 is induced by UV light or ADR, but not by IR (Kapoor and Lozano, 1998; Lu et al., 1998). Serines 376 and 378 in the C-terminal region are reported to be constitutively phosphorylated, and treatment with IR led to the dephosphorylation of serine 376 (Waterman et al., 1998). Phosphorylation of serines 315 and 392 affects the oligomerization state of p53 (Sakaguchi et al., 1997) and its ability to bind DNA in a sequence-specific manner, at least *in vitro* (Hupp et al., 1992; Wang and Prives, 1995; Hao et al., 1996). Thr155 and Thr150 or Ser149, in the central, site-specific, DNA-binding domain, recently were reported to be phosphorylated by the COP9 signalosome (CSN)-associated kinase (Bech-Otschir et al., 2001); so far, these are the only sites in the central domain that have been reported to be posttranslationally modified. In fission yeast, the COP9 signalosome is required for the activation of ribonucleotide reductase (Nielsen, 2003); in mammals, it also may participate in regulating p53 degradation.

Acetylation of the p53 C terminus is mediated through a DNA damage initiated, phosphorylated-dependent signaling cascade by the histone acetyltransferases and transcriptional coactivators p300, CBP, and PCAF (Gu and Roeder, 1997; Sakaguchi et al., 1998; Prives and Manley, 2001). The interaction of p53 with p300/CBP was shown to be enhanced by phosphorylation of p53 at serine 15 (Lambert et al., 1998; Dumaz and Meek, 1999); in turn, CBP/p300 acetylates several C-terminal lysines including 372, 373, 381, and 382. Recently, lysine 305 also was shown to be acetylated in response to IR, UV, H_2O_2, and actinomycin D (Wang et al., 2003). Peptide competition experiments suggest that phosphorylation of Thr18 and Ser20 also may enhance the recruitment of CBP/p300 to p53 (Dornan and Hupp, 2001); however, Saito et al. found that acetylation of Lys382 was decreased by mutations that changed Ser6, 9, 15, or Thr18, but not Ser20 or more distal sites, to alanine (Saito et al., 2002), presumably by inhibiting phosphorylation at these sites. The acetylated C-terminal lysines also are targets for ubiquitination; thus, acetylation may directly contribute to p53 stabilization (Nakamura et al., 2002). Lysine 386 is reported to be sumoylated, although only at low levels (Melchior and Hengst, 2002).

The availability of modification-specific antibodies has allowed a detailed characterization of the phosphorylation and acetylation of p53 in cultured human cells following exposures to genotoxic agents, including IR, UV, adriamycin (Saito et al., 2003), or nitrogen oxide (NO) (Hofseth et al., 2003), as well as to non-genotoxic agents, including the presence of activated oncogenes (e.g. *Ras*) (Bulavin et al., 2002b), microtubule disruptors (taxol, nocodazole), nucleoside synthesis inhibitors (PALA) (Saito et al., 2003), hypoxia (Hammond et

al., 2002), and osmotic stress (Kishi et al., 2001). Use of these antibodies, most of which are now commercially available, coupled with cell lines defective in one or more signaling enzymes or the use of highly specific chemical inhibitors, has begun to elucidate the pathways that lead to specific p53 modifications. Such studies also have revealed some unexpected relationships.

In response to DNA double-strand breaks (DSBs), one of the earliest modifications to p53 that can be detected is phosphorylation of serine 15 (Siliciano et al., 1997). Although serine 15 was first identified as a site phosphorylated *in vitro* by the DNA-dependent protein kinase, DNA-PK (Lees-Miller et al., 1992), later it was shown that DNA-PK was not required to phosphorylate this site *in vivo*, nor was DNA-PK needed for the physiological responses to DNA damage that depend on p53 (Jimenez et al., 1999). DNA-PK is a member of a small family of large protein kinases that more closely resemble phosphatidylinositol-3 kinases (PI3K) in their kinase domains than the majority of serine/threonine kinases, and DNA-PK was found to preferentially phosphorylate serines or threonines that were followed by glutamine, the so-called SQ/TQ motif (Anderson and Lees-Miller, 1992). In mammalian cells, the PI3K-like kinase family includes four additional members, ATM (ataxia telangiectasia (A-T) mutated), ATR (A-T and RAD3 related), FRAP (FK506 binding protein12-rapamycin associated protein kinase), and SMG1 (also called ATX), a recently described protein kinase involved in nonsense-mediated mRNA decay (Denning et al., 2001; Yamashita et al., 2001). Each of these kinases also recognizes SQ/TQ motifs in protein substrates, and each phosphorylates serine 15 of p53 (or in a p53-related peptide) *in vitro* (Abraham, 2001). FRAP is involved in the regulation of translation initiation in response to nutrients and growth factors, but its activity also increases at late times

after exposure of cells to UV light, where it transmits a signal for the production of immunosuppressive cytokines (Yarosh et al., 2000). Whether SMG1/ATX, FRAP or DNA-PK are ever important for phosphorylating p53 or regulating its activity *in vivo* is unclear; however, ATM and ATR are both believed to directly phosphorylated p53 on Ser15 *in vivo* in response to DNA damage (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Cell lines that lacked ATM or that overexpressed a dominant-negative allele of ATR are deficient in p53 phosphorylation at Ser15 *in vivo* and are defective in the activation of DNA damage-induced cell cycle checkpoints (Abraham, 2001; Abraham, 2003; Shiloh, 2003).

Studies using phospho-specific antibodies and cell lines deficient in ATM revealed that phosphorylation of p53 at Ser9, Thr18, Ser20, and Ser46 are dependent on the ATM kinase (Saito et al., 2002). These sites, all of which are phosphorylated in response to DNA damage *in vivo*, do not correspond to the SQ/TQ motif and are not believed to be phosphorylated by ATM or ATR directly. Rather, phosphorylation of these sites is believed to depend on effector kinases that are activated in response to ATM or ATR, or that require phosphorylation of Ser15 for recognition of p53. Two protein kinases capable of phosphorylating Ser46, p38 MAPK (Bulavin et al., 1999) and HIPK2 (D'Orazi et al., 2002; Hofmann et al., 2002), both of which are activated after exposure of cells to UV light, have been described; however, neither has been shown to be ATM dependent. Serines 6 and 9 became strongly phosphorylated in response to both IR- and UV-induced DNA damage, which indicates that Ser9 could be phosphorylated by CK1 or a CK1-like kinase in response to phosphorylation of Ser6 (Higashimoto et al., 2000). *In vitro* CK1 phosphorylates serines and threonines two residues distal to a phosphorylated serine or

threonine. However, in response to IR, phosphorylation of Ser9 appears to be independent of phosphorylation at Ser6; thus, phosphorylation of Ser9 appears to be dependent upon activation of an unknown protein kinase that is activated by ATM. Alternatively, recognition of p53 by this kinase requires phosphorylation of p53 at Ser15.

Recently, using mutant p53s in transient transfection experiments in which individual serines were changed to alanines, Saito et al. (2003) demonstrated additional N-terminal p53 phosphorylation sites interdependencies. As had been shown previously (Bulavin et al., 1999), changing Ser33 to alanine blocked phosphorylation of Ser37, but changing Ser37 to alanine had no effect on phosphorylation at Ser33 or at other N-terminal sites. Changing Ser6 to alanine blocked phosphorylation at Ser9 and vice versa without affecting phosphorylation at the other N-terminal sites. Most strikingly, substituting alanine for Ser15 prevented IR-induced phosphorylation at Ser9, Thr18, and Ser20, while phosphorylation of Ser6, Ser33, Ser37, and Ser46 were unaffected. Similarly, changing Ser20 to alanine prevent phosphorylation of Thr18, while changing Thr18 to alanine reduced phosphorylation at Ser20 but not at the other N-terminal sites. Changing Ser37 or Ser46 to alanine had no significant effect on the phosphorylation of other sites, nor did phosphorylation of the C-terminal sites, Ser315 or Ser392, depend on any of the N-terminal phosphorylation sites or vice versa. Control experiments suggested that changing serine to alanine did not prevent recognition by phospho-specific antibodies. Thus, on the basis of single-site mutant analyses, the N-terminal p53 phosphorylation sites can be classified into four clusters: Ser6 and Ser9; Ser9, Ser15, Thr18, and Ser20; Ser33 and Ser37; and Ser46. Furthermore, phosphorylation of the Ser15 cluster (Ser9, Ser15, Thr18,

and Ser20) appears to require DNA damage (Saito et al., 2003). Presently, it cannot be determined whether phosphorylation at dependent sites requires a nearby serine or the phosphorylation of that serine (or threonine); nevertheless, these results suggest that at least some site interdependencies reflect mechanisms that permit signal amplification and the integration of information from diverse signaling pathways by requiring sequential phosphorylation of sites in an ordered manner. For example, Ser9, Thr18, and Ser20 will not be phosphorylated unless Ser15 is first phosphorylated, and Ser15, Thr18, and Ser20 may all be required for efficient p53 stabilization. Furthermore, this intramolecular cascade mechanism might serve to check inappropriate p53 activation or regulate the intensity of the p53 response and would complement kinase activation cascades (Saito et al., 2002).

4. Signaling to p53

The mechanisms by which cells detect genotoxic and non-genotoxic stresses and signal to p53 are complex and still incompletely understood. However, phosphorylation of p53 in response to DNA damage appears to be principally driven by two related signaling pathways, one mediated by ATM, the other by ATR, that are activated by different mechanisms in response to different DNA insults.

4.1 ATM-dependent signaling to p53

Although there are many different forms of DNA damage, among the most dangerous are DNA double-strand breaks (DSBs). DNA DSBs result from exposure to external insults such as ionizing radiation and treatments with certain anti-cancer agents; it has been estimated, however, that even in the absence of exposure to genotoxic substances each human cell undergoes approximately eight DSBs per day from physical forces and oxidative damage generated in the course of normal cellular metabolism (Bernstein and Bernstein, 1991). While the consequences of naturally occurring DSBs were probably the evolutionary driver for development of systems that all cells have for recognizing DSBs and taking appropriate actions, treatment with ionizing radiation, radiomimetic drugs (e.g. neocarzinostatin (NCS), or toposiomerase II inhibitors (e.g. adriamycin or etoposide) frequently is used in the laboratory to study the consequences of DSBs. It must be remembered, however, that these agents have other effects. For example, IR produces far more single-stranded breaks and cluster damaged sites than simple DSBs (Sutherland et al., 2000).

In mammalian cells, cellular responses to DSBs, including phosphorylation of p53 at several sites, are heavily dependent upon the ATM protein kinase. Loss of ATM function in humans causes ataxia telangiectasia (A-T), a devastating disease characterized by progressive neurodegeneration, immunodeficiency, sterility, and a high risk of cancer (Shiloh, 2003). A-T cells are hypersensitive to killing by ionizing radiation but show normal sensitivity to UV light. While our understanding of the complex mechanism(s) by which DSBs activate ATM are incomplete, remarkable progress recently has been made. Immediately after exposure of cells to

IR or radiomemetic agents, a moderate but reproducible increase in ATM kinase activity can be measured in immune complex assays (Banin et al., 1998; Canman et al., 1998). This increased activity is not accompanied by changes in ATM abundance or subcellular distribution. Purified ATM was shown to interact preferentially with the ends of double-stranded DNA fragments (Smith et al., 1999; Suzuki et al., 1999), but DNA is not required to sustain ATM activity in immune complexes; thus, the implications of this finding with respect to activation *in vivo* remain unclear. Nevertheless, a small fraction of the ATM molecules in cells became resistant to extraction and were detected as nuclear aggregates immediately following the induction of DSBs (Andegeko et al., 2001). Furthermore, the retained fraction of ATM colocalized with the phosphorylated form of histone H2AX (γ-H2AX) and with foci of the Nbs1 protein, suggesting that ATM associates with DSBs. DSB-induced γ-H2AX foci appear before those of most other proteins that form foci after DNA damage, and the number of γ-H2AX foci is proportional to the number of induced DSBs (Paull et al., 2000; Schultz et al., 2000; Bonner, 2003). γ-H2AX is phosphorylated at serine 139, an SQ site, by the ATM kinase *in vitro*, and ATM is necessary for this phosphorylation *in vivo* early after the induction of DSBs (Burma et al., 2001). Together, these results indicate that ATM is activated very early after DSB induction at or near the sites of DNA double-strand breaks.

A hint as to the mechanism of activation came from work in Lavin's laboratory which showed that ATM from unirradiated cells was activated in the absence of DNA after preincubation with ATP (Kozlov et al., 2003). Activation required Mn^{2+}, a required ATM

cofactor, and was inhibited by wortmannin, a PI3K-specific inhibitor. Activation was reversed by phosphatase treatment, suggesting that activation involved autophosphorylation. Then, in a technical *tour de force*, Bakkenist and Kastan (2003) identified Ser1981, which resides in the sequence GSQS N-terminal to the kinase domain, as an IR-inducible phosphorylation site in the ATM polypeptide. Using a phospho-Ser1981-specific antibody, they then showed that a kinase-dead ATM mutant was phosphorylated in IR-treated cells that contained wildtype ATM but not in A-T cells that lack functional ATM, but this mutant was not phosphorylated in cells that expressed the related PI3Ks ATR and DNA-PK (Bakkenist and Kastan, 2003). This result strongly suggests that Ser1981 is phosphorylated as the result of self- or auto-phosphorylation. Ser1981 resides near the N terminus of a FAT (FRAP, ATM, and TRRAP) domain, a ~500 amino acid region found only in PI3K-related proteins that may serve as a structural scaffold or as a protein-protein interaction domain (Bosotti et al., 2000). Subsequent analysis of ATM protein fragments showed that the kinase domain and the FAT domain stably bound one another and that the sequences flanking Ser1981 are important for this interaction. However, mutating Ser1981 to aspartic or glutamic acid, which mimic serine phosphorylation, prevented interaction of the FAT domain with the kinase domain, suggesting that autophosphorylation results in the dissociation of a complex containing two or more inactive ATM molecules. These findings are consistent with a model in which ATM is activated in response to DSBs by autophosphorylation at Ser1981, which results in a dissociation of the ATM complex into monomers that are then capable of interacting with substrates (Fig. 3).

Although the above model superficially fits expectations, the astonishing finding of

Bakkenist and Kastan (Bakkenist and Kastan, 2003) is that the majority of ATM molecules in a cell became activated within a few minutes after exposure to IR doses that produce only a few DSBs per cell. At these low doses (0.1 Gy, which is expected to produce ~4 DSBs/cell), it is inconceivable that each ATM molecule can associate with a DSB as a requirement for activation within the time that was available. To explain this observation, Bakkenist and Kastan proposed that a DSB could reveal its presence by triggering a relatively widespread change in chromatin structure with which ATM could interact to trigger conversion of inactive ATM complexes into active monomers through autophosphorylation. Consistent with this hypothesis, the authors indeed found that treatment of cells with a histone deacetylase inhibitor induced phosphorylation on Ser1981 and resulted in the concomitant phosphorylation of p53 on Ser15. This finding suggests that activation of p53 in response to DSBs is a two-stage process (Fig. 3). First, a fraction of the nuclear ATM interacts with DSBs or other sensor proteins such as MRN (Mre11, Rad50, Nsb1) or the Rad17 complexes that rapidly bind to DSBs. Indeed, recent results show that the MRN complex is required for proper activation of ATM (Uziel et al., 2003). The tightly bound fraction of ATM is activated by autophosphorylation and rapidly phosphorylates H2AX and other proteins that assemble at DSB sites, recruiting additional proteins to the DSB sites. The assembled complex then triggers a change in chromatin conformation over a distance of perhaps a megabase which, in turn, provides a larger target for the interaction of additional, free ATM complexes that then autophosphorylate to become active, free monomers. The activated, free ATM molecules rapidly phosphorylate effector kinases, such as Chk2, and other substrates, e.g. p53, Mdm2, BRAC1, to accomplish control of cell cycle progression and activation of DNA repair and perhaps apoptosis. Although this model has considerable appeal, several questions

remain. How does ATM sense both DNA ends and changes in chromatin structure? What is the nature of the change in chromatin structure, and how is this change distinguished from changes that accompany chromatin remodeling associated with normal transcription and DNA replication?

4.2 ATR-dependent signaling to p53

Activation of ATR, the ATM and RAD3-related kinase, is not as well understood as activation of ATM, in part because inactivation of ATR results in lethality, and only recently have genetic constructs been engineered that allow the consequences of ATR activation to be deduced at the molecular level (e.g. (Cortez et al., 2001; Zou et al., 2002)). ATR is activated after exposure of cells to UV light or alkylating agents, which produce bulky lesions in DNA, or treatment with anti-cancer drugs (e.g. adryamycin), hydroxyurea, or extreme hypoxia that may block transcription or replication or cause replication fork collapse (Abraham, 2001; Hammond et al., 2002; Brown and Baltimore, 2003). ATR also is activated at later times after the creation of DSBs, which probably accounts for delayed phosphorylation of p53 at Ser15 in A-T cells (Saito et al., 2002). However, it is unclear whether the DNA damage that leads to ATR activation is sensed directly or whether ATR is responding to a consequence of blocked transcription or replication or both (Fig 4). As for ATM, activation of ATR is not accompanied by changes in ATR abundance or subcellular distribution. Unlike ATM, ATR isolated from cells treated with DNA damage-inducing agents does not display increased activity in kinase assays *in vitro* (Tibbetts et al., 2000). Furthermore, neither ATR nor the other PI3K-like kinases (DNA-PK or

FRAP) have an SQ/TQ site at the N terminus of their FAT domains equivalent to the GSQS Ser1981 autophosphorylation site in ATM (Bosotti et al., 2000), making autophosphorylation less likely as a mechanism for ATR activation in response to DNA damage.

In mammalian cells, ATR exists as a stable complex with ATRIP (ATR interacting protein), an 85 kDa protein that stabilizes ATR and may help regulate its activity (Cortez et al., 2001). *In vitro*, ATR phosphorylates ATRIP, and both proteins colocalize to intranuclear foci that may correspond to sites of DNA synthesis and repair. Recent studies by Zou and Elledge show that replication protein A (RPA), a protein complex that associates with single-stranded DNA (ssDNA) and becomes highly phosphorylated on its 34 kDa subunit following DNA damage, is required to recruit ATR-ATRIP to sites of DNA damage and to form nuclear foci. *In vitro*, RPA stimulated the binding of ATRIP to single-stranded DNA and the phosphorylation of Rad17 on Ser635, an *in vitro* and *in vivo* site of phosphorylation by ATR. RPA also was required for ATR-mediated activation of the Chk1 kinase in human cells. These studies suggest that ssDNA may be a common intermediate that functions as a signal for activation of ATR-ATRIP (Fig. 4). Single-stranded gaps are generated as an intermediated in the repair of bulky lesions by nucleotide excision repair. When replication forks encounter DNA lesions, longer stretches of ssDNA could be generated by the stalling of polymerases and/or the uncoupling of helicases and polymerases. Thus, Zou and Elledge suggest that the apparent activation of ATR may be achieved by the simultaneous enrichment of ATR-ATRIP complexes and their substrates at sites of DNA damage (Zou et al., 2002). If this is the case, it will be interesting to see whether proteins besides RPA target ATR for colocalization with substrates.

5. Non-genotoxic stress and p53 effector kinases

ATM and ATR both phosphorylate p53 at Ser15 *in vitro*, and elimination of Ser15 prevents p53 phosphorylation by ATM (Banin et al., 1998; Canman et al., 1998), indicating that other kinases are responsible for phosphorylating p53 at other sites (Fig. 2). In response to DSBs, ATM activates the Chk2 kinase through phosphorylation of Thr68, and Thr68 is required for the full activation of Chk2 in response to IR (Ahn et al., 2000; Melchionna et al., 2000). Likewise, Chk1 is phosphorylated and activated in response to UV light *in vivo* in an ATR-dependent manner, and *in vitro* ATR phosphorylates Chk1 on serine 317 and 345 (Zhao and Piwnica-Worms, 2001). Early studies by Shieh et al. (2000) and Chehab et al. (2000) reported that Chk1 and Chk2 phosphorylated p53 at Ser20, and possibly other sites, resulting in its stabilization and activation in response to DNA damage. These results are consistent with a requirement for ATM for the phosphorylation of Ser9, Thr18, and Ser20 in response to IR (Saito et al., 2002); however, several recent studies question the role of the Chk2 effector kinase in mediating p53 phosphorylation at Ser20 as well as the role of Ser20 in stabilizing and activating p53. First, in contrast to changing Ser18 of murine p53 (the equivalent of Ser15 in human p53) to alanine (Chao et al., 2000), Wu et al. found that changing Ser23 (Ser20 in human p53) to alanine had no effect on p53 stability or activity in mouse ES cells, fibroblasts or thymocytes (Wu et al., 2002). Second, Takai et al. showed that mouse p53 Ser23 and human p53 Ser20 were phosphorylated equally well in cell from wildtype or Chk2 knockout mice, although p53-mediated transactivation of several target genes was abolished (Takai et al., 2002). The lack of a need for Chk2 to phosphorylate p53 Ser20 recently was confirmed by Jallepalli et al. (2003). Third,

reexamination of p53 phosphorylation *in vitro* by purified Chk2 indicated that p53 was a weak substrate compared to Cdc25C (Ahn et al., 2003). Furthermore, inhibition of Chk2 expression with small, interfering RNAs (siRNA) led to a marked reduction in Chk2 protein, but p53 was still stabilized and active as a transcription factor. Similar results also were seen with siRNA-mediated targeting of Chk1, suggesting that neither Chk1 nor Chk2 regulate p53 stability or activity. Together with the recently reported interdependence of p53 phosphorylation at Ser9, Ser15, Thr18, and Ser20 (Saito et al., 2003), these results indicate that the role of Ser20 in stabilizing p53 should be re-evaluated.

In contrast to the Ser15 cluster, most other known phosphorylation sites in p53 (except Ser37) are phosphorylated in response to both genotoxic and non-genotoxic stresses (Saito et al., 2003). With the exception of Ser6 and 9, kinases that can phosphorylate most of these sites *in vitro* have been reported (Fig. 2); however, in most cases, to date there is little compelling evidence that these kinases phosphorylate p53 *in vivo*. Furthermore, for the most part it is not known if or how these kinase are activated in response to various forms of cellular stress.

After Ser15, a second important phosphorylation site is Ser46. Serine 46 of human p53 was shown to be phosphorylated in cells exposed to UV light (Bulavin et al., 1999). *In vitro*, Ser33 and Ser46 were phosphorylated by the p38 MAP kinase, and mutation of both these sites decreased p53-mediated and UV-induced apoptosis. Ser46 also was shown to be required for induction of p53AIP1, a mitochondrial localized protein whose enhanced expression leads to cell death (Oda et al., 2000). Subsequently, two laboratories showed that homeodomain-interacting

protein kinase-2 (HIPK2) was activated after exposure of cells to UV light; HIPK2 also phosphorylated p53 on Ser46 *in vitro* (D'Orazi et al., 2002; Hofmann et al., 2002). Furthermore, HIPK2 interacts with and colocalizes with p53 and CBP in PML nuclear bodies, thus facilitating p53 acetylation. As noted above, Ser46 also is phosphorylated after IR, and phosphorylation in response to IR is ATM dependent (Saito et al., 2002); however, it is unclear if either p38 MAPK or HIPK2 can be activated by ATM. Interest in the potential role of p38 MAPK in regulating p53 activity recently was stimulated by the finding that the gene (*PPM1D*) for Wip1, a p53-induced protein phosphatase that negatively regulates p38 MAPK activity (Fiscella et al., 1997; Takekawa et al., 2000), is amplified in 12 to18 percent of primary human breast cancers (Bulavin et al., 2002b; Li et al., 2002). Wip1 thus forms a negative feedback loop with p53 analogous to the p53-Mdm2 feedback loop. Amplification of the Wip1 gene in cancers, which would inhibit p38 MAPK-mediated activation of p53 through phosphorylation of Ser33 and Ser46, is consistent with a role for p38 MAPK in regulating p53 activity *in vivo*.

A large number of proteins have been shown to interact with p53, at least *in vitro*, and, as shown in figure 2, most of these interact with the N- or C-terminal regions of p53 that are both unstructured and become highly modified in response to stress. This coincidence is unlikely to be accidental. Rather, it seems highly probably that the interaction of some of these and other proteins will be enhanced or inhibited by p53 posttranslational modifications. In turn, the complexes thus formed are likely to modulate p53 function and regulate cell fate. To date, the interaction with p53 of only a few of the proteins listed in figure 2 have been shown to be modulated by phosphorylation. As described above, foremost among these are the HATs,

p300/CBP. The role of phosphorylation in regulating the interaction of p53 and Mdm2 is still controversial (Schon et al., 2002; Anderson and Appella, 2003). Nevertheless, the roles for phosphorylation and acetylation in modulating interactions of proteins with p53, including protein kinases, HATs, HDACs, and their adaptors, will be a fruitful area for future research.

6. Conclusions

Cellular responses to both genotoxic and non-genotoxic stress are complex and involve multiple signaling pathways. This is well illustrated by the p53 tumor suppressor protein, which itself represents but one node in the cellular pathways that regulate cell function in response to both internal and external stimuli. Studies over the past 20 years have elucidated most of the posttranslational modifications to p53 that, in turn, modulate its stability and activity. The availability of reagents (antibodies) that are highly specific for p53 modified at specific sites, coupled with new genetic techniques for abrogating gene function, is facilitating elucidation of multiple, interacting pathways that posttranslationally modify p53 through phosphorylation or acetylation. Stress *signals* much first be detected through some change, the binding of a ligand to a membrane receptor or the recognition of new or unusual internal structures (e.g. DSBs or pyrimidine dimers) by *sensors* (Petrini and Stracker, 2003). Such structures may require processing by *signal modifiers* (D'Amours and Jackson, 2002), e.g. the excision of dimers leaving a region of single stranded DNA, to allow recognition by the proximal *signal transducers*, which usually are protein kinases (e.g. ATM, ATR, p38 MAPK) (Abraham, 2001;

Bulavin et al., 2002a; Shiloh, 2003). Signal recognition by signal transducers may require *adaptors* (e.g. RPA) to recognize proximal processed signals (ssDNA), and *mediators* (e.g. Rad9, Mdc1) (Canman, 2003) to transmit signals to effectors (e.g. Chk1, Chk2) that ultimately modify targets, such as p53. p53 then integrates signal strength and/or signals from several sources to ultimately determining cell fate through the induction or repression of specific genes, or by direct interaction with components that mediate apoptosis. Signaling pathways often are branched and interconnected. Likewise signals, especially external environmental signals, may not be pure, thereby activating more than one signaling pathway. While substantial progress has been made in characterizing the pathways that respond to DNA damage and signal to p53, these pathways are still incompletely characterized and the actual mechanisms that detect DNA damage are only now becoming clear. Nevertheless, thank in part to new technologies, rapid progress can be expected over the next few years.

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Figure Legends

Figure 1. Signaling pathways for the activation of p53. The p53 tumor suppressor is stabilized and activated as a transcription factor in response to several signaling pathways that are initiated in response to genotoxic damage or non-genotoxic cellular stresses. Depicted schematically are the major genotoxic pathways that respond to DNA double-strand breaks through activation of ATM, to bulky lesions in DNA that block transcription or DNA replication and signal through ATR, and non-genotoxic stress pathways that generally do not involve ATM or ATR but signal through p38 MAPK and other signaling systems. ATM and ATR directly phosphorylate several DNA damage associated proteins including BRCA1, 53BP1, H2AX, and p53 as well as several effector protein kinases, such as Chk1 and Chk2. The response to extreme hypoxia is exceptional in that the resulting collapsed replication forks are believed to activate ATR, resulting in the phosphorylation of p53 at Ser15 but not its subsequent acetylation at Lys382 (Hammond et al., 2002).

Figure 2. Protein domains, posttranslational modification sites, and proteins that interact with human p53. The 393 amino acid, human p53 polypeptide is represented schematically (box) with the five most highly conserved regions marked (I-V); postulated function regions and domains also are indicated. Residues ~1-42 comprise the transactivation domain; residues ~63-97 constitute a Src homology 3-like (SH3) domain that overlaps a poorly conserved proline and alanine rich segment (33-80); residues ~102-292 contain the central, sequence-specific, DNA binding core region; residues ~300-323 contain the primary nuclear localization signal (NLS);

residues 324-356 comprise the tetramerization domain (TET) which contains a nuclear export signal; residues 363-393 (REG) negatively regulate DNA binding by the central core to consensus recognition sites in oligonucleotides and interact in a sequence-independent manner with single- and double-stranded nucleic acids. Interaction regions for selected proteins are indicated below the polypeptide, and posttranslational modification sites (P, phosphorylation; Ac, acetylation) are indicated above the peptide together with enzymes that can accomplish the modifications *in vitro*. Lys386 may be modified by conjugation with SUMO1, a ubiquitin-like peptide. References are found in the text and recent reviews, e.g. (Ko and Prives, 1996; Anderson and Appella, 2003; Craig et al., 2003).

Figure 3. Activation of p53 in response to DNA double-strand breaks. In step 1, DNA DSBs result in the rapid activation through autophosphorylation of a fraction of a cell's ATM. This fraction becomes tightly associated with chromatin through ATM's DNA end-binding activity (Smith et al., 1999; Suzuki et al., 1999); it then phosphorylates H2AX and perhaps other substrates that assemble at the break site (Redon et al., 2002; Shiloh, 2003). H2AX is a variant of H2A with a C-terminal extension that can be directly phosphorylated (yellow circles) by ATM. It is found with RAD9, RAD1, RAD17, HUS1, and the MRN complex (Mre11, Rad50, Nbs1) in foci of DNA damage sensors and repair proteins that form at DSBs sites after DNA damage (D'Amours and Jackson, 2002; Fei and El-Deiry, 2003; Petrini and Stracker, 2003). The DSB induces a change in chromatin conformation, with which the bulk of a cell's ATM interacts to become activated, also through autophosphorylation in step 2 (Bakkenist and Kastan, 2003). Autophosphorylation at Ser1981 causes ATM to dissociate into active monomers. ATM directly

phosphorylates Ser15 near the N terminus of p53 and is required for the phosphorylation of Ser9, Ser20, Ser46, and Thr18, presumably as a consequence of ATM-dependent activation of effector protein kinases (Saito et al., 2002) and/or creation of kinase recognition sites (Saito et al., 2003). Phosphorylation of Mdm2 and p53 may promote dissociation of p53 and Mdm2, inhibits p53 degradation, and promote association of p53 with its coactivator p300/CBP. However, association of p300/CBP with the p53/Mdm2 complex may promote p53 multi-ubiquitination and its degradation through the 26s proteosome. ATM also phosphorylates other substrates including BRCA1, 53BP1, Mdm2, and downstream effector kinases, such as Chk2.

Figure 4. Activation of ATR in response to blockage of transcription by RNA polymerase II and the arrest of DNA replication. ATR is activated in human cells in response to UV radiation and chemicals that produced bulky lesions and oxidized DNA bases. These, in turn, may block RNA transcription by RNA polymerase II (poli) and DNA replication. In human cells, ATR exists in a stable complex with ATRIP (ATR-interacting protein) (Cortez et al., 2001). ATR is recruited to sites of DNA damage that contain single-stranded DNA segments through the interaction of ATRIP with RPA (Zou and Elledge, 2003), suggesting that RPA-ssDNA, a complex common to several DNA repair processes, may serve as a DNA damage signal for the recruitment of ATR-ATRIP. In contrast to ATM, ATR isolated from cells exposed to DNA damaging agents does not display increased kinase activity (Tibbetts et al., 2000); thus, "activation" may be achieved by the simultaneous recruitment of ATR-ATRIP and substrates to sites of DNA damage (Zou and Elledge, 2003). ATR activates the effector kinase Chk1 and is believed to phosphorylated p53 at Ser15 and Ser37. As noted above, extreme hypoxia does not cause detectable DNA damage but

is believed to activate ATR by causing the collapse of DNA replication forks (Hammond et al., 2002).

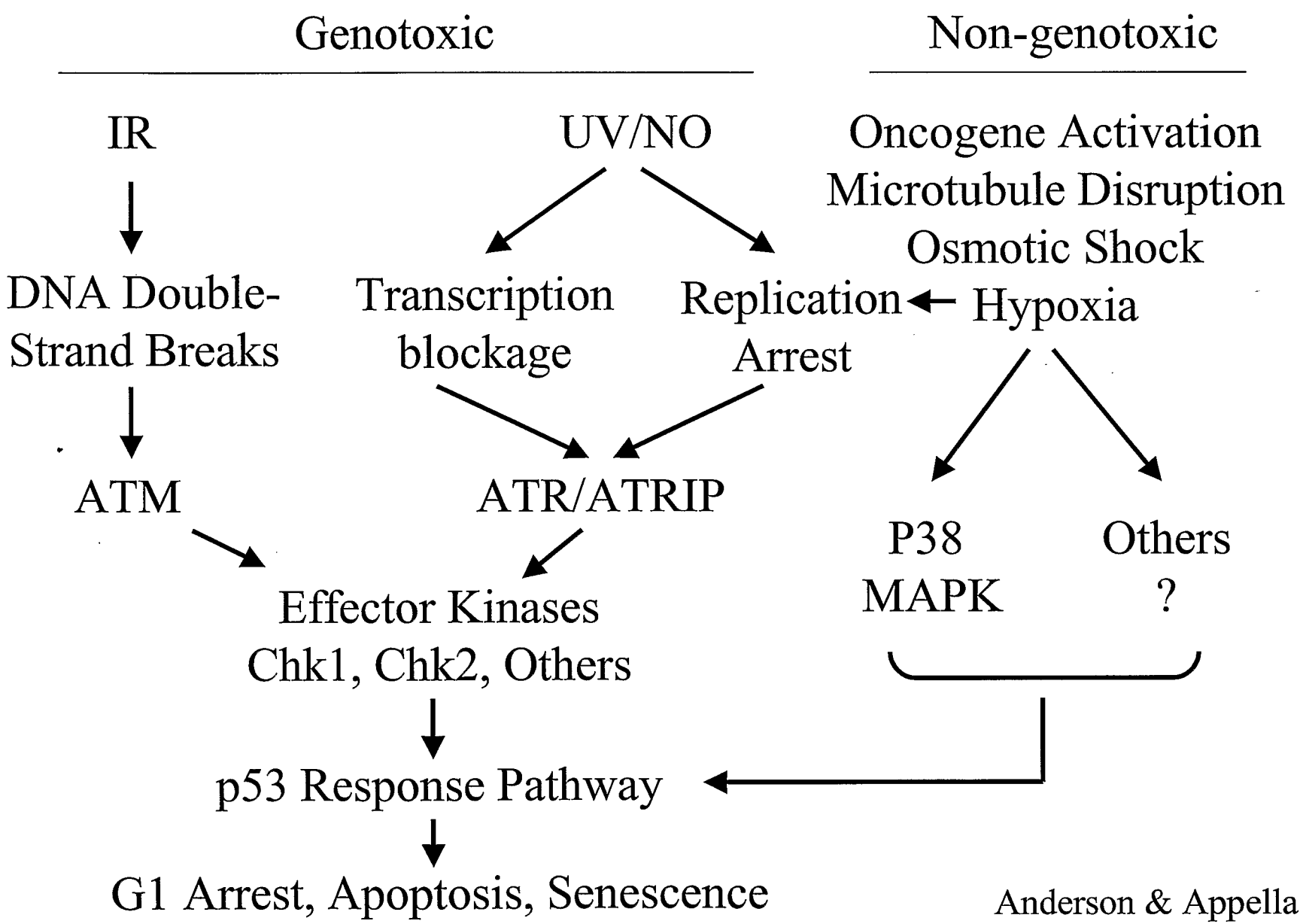

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Figure 1 9/11/03

Human p53 Tumor Suppressor Protein

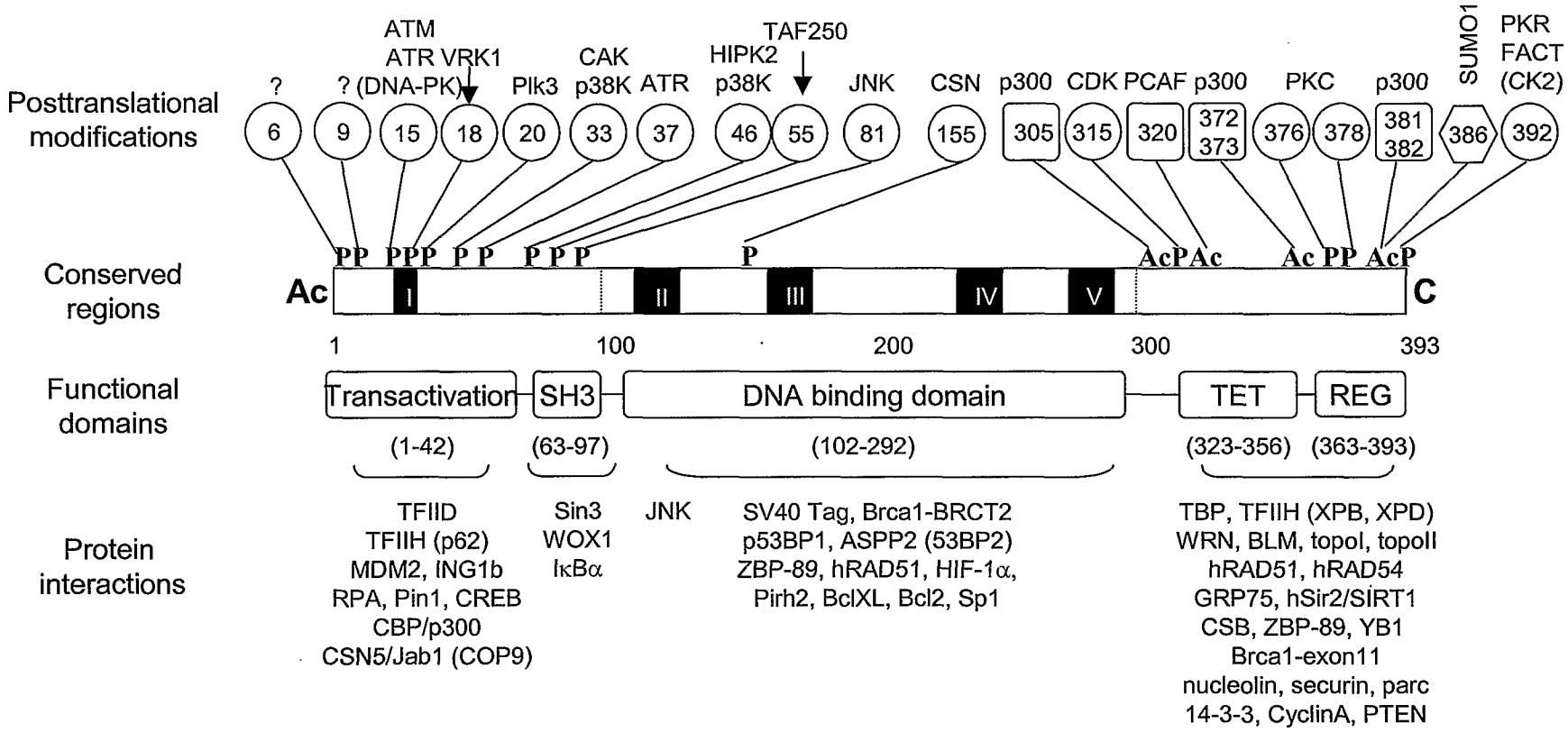

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Figure 2, 9/10/03

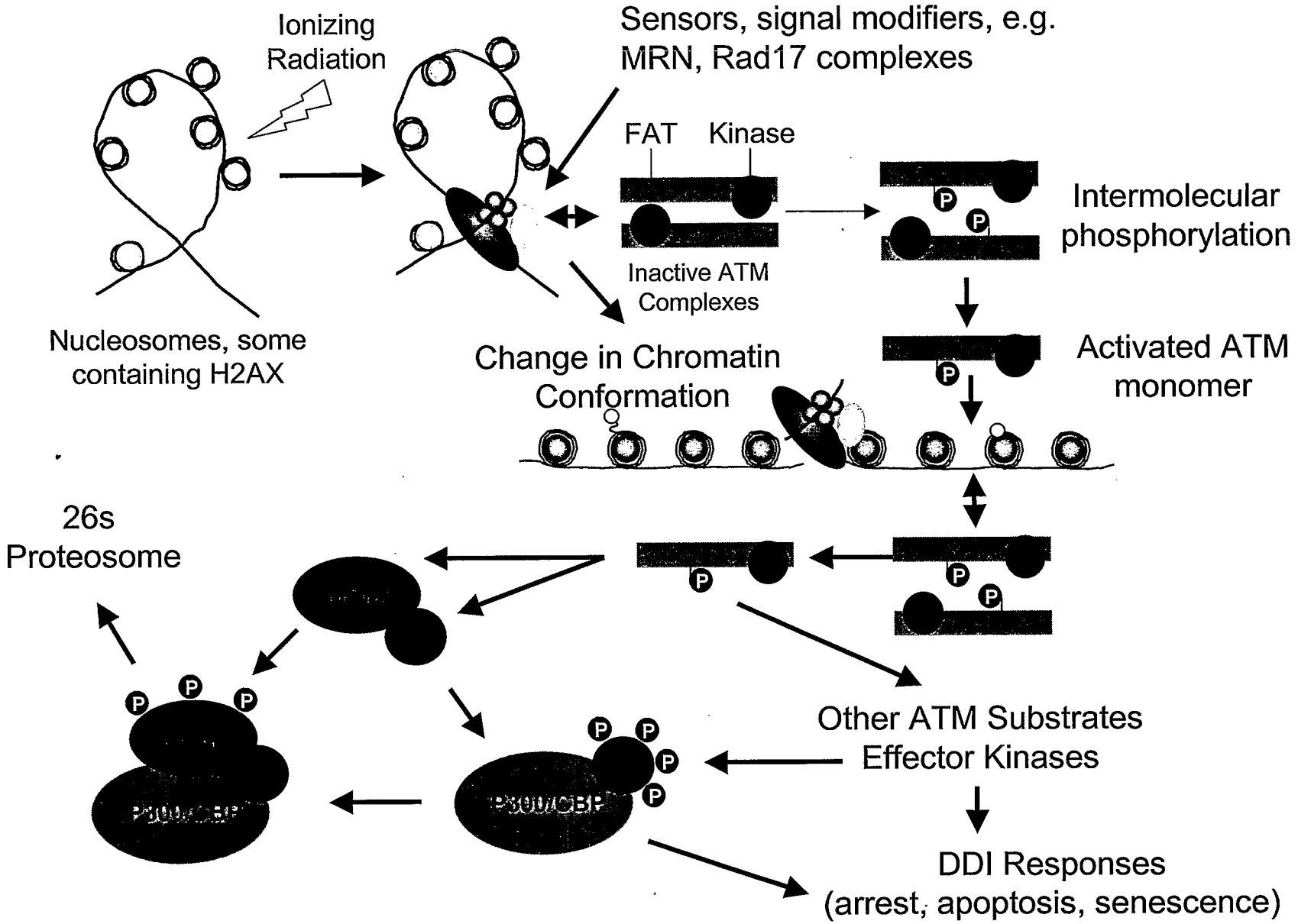

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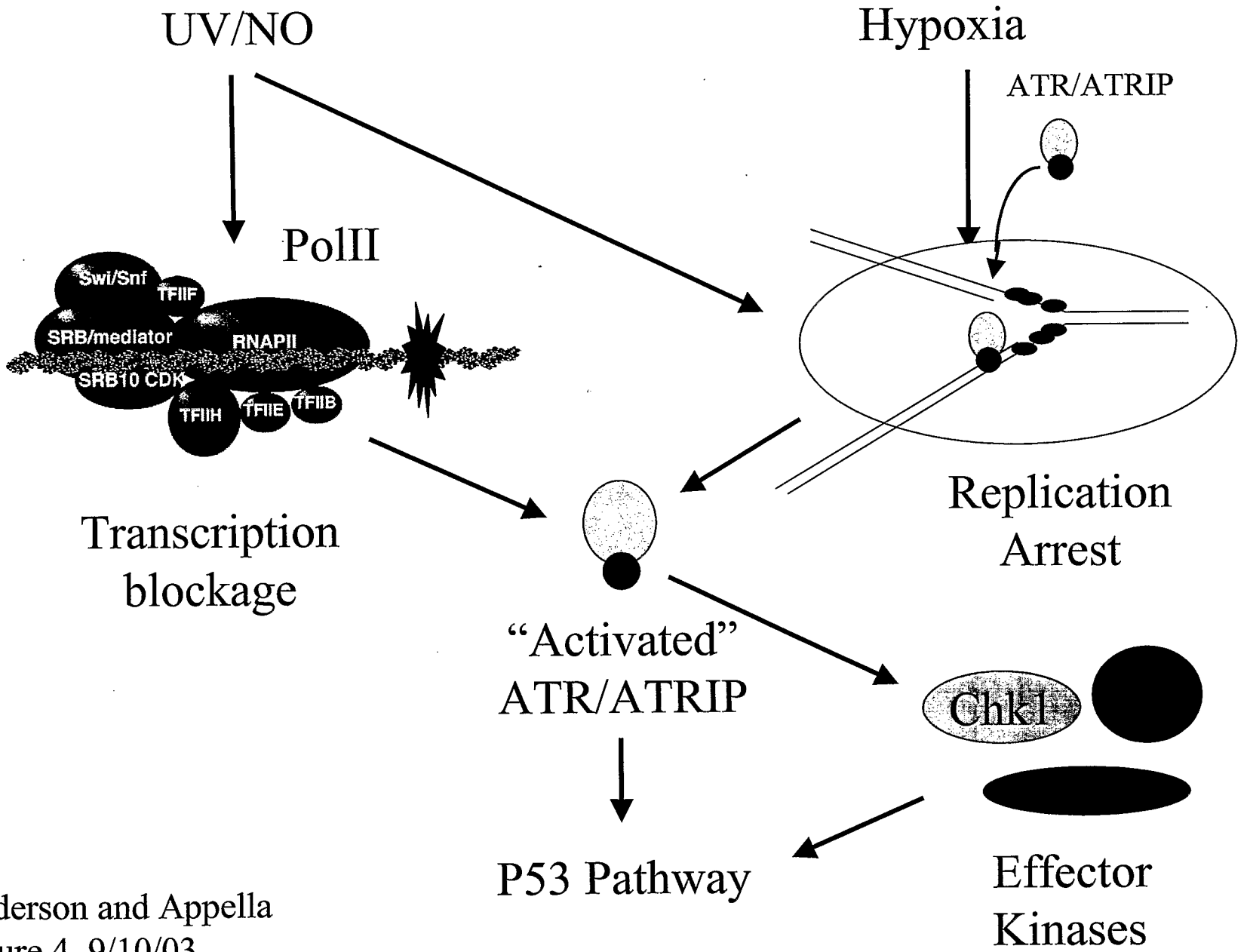
Figure 3 10/11/03



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